Kidney International, Vol. 63 (2003), pp. 534–542

Induction of DNA synthesis by ligation of the CD53 tetraspanin antigen in primary cultures of mesangial cells

Mónica Yunta, Alicia Rodríguez-Barbero, Miguel A. Arévalo, José M. López-Novoa, and Pedro A. Lazo

Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas; Departamento de Fisiología y Farmacología, Instituto Reina Sofía de Investigaciones Nefrológicas; and Departamento de Anatomía e Histología Humanas, Universidad de Salamanca, Campus Miguel de Unamuno, Salamanca, Spain

Induction of DNA synthesis by ligation of the CD53 tetraspanin antigen in primary cultures of mesangial cells.

Background. The interaction of mesangial cells with the extracellular matrix plays a major role in kidney biology. Tetraspanin proteins modulate cell interaction with the extracellular matrix. Tetraspanins form supramolecular structures on the cell membrane that send signals after engagement by unknown ligands, modulate different signaling processes, and regulate cell adhesion and motility.

Methods. CD53 was determined by immunohistochemistry, and on the cell surface of cultured rat mesangial cells by flow cytometry. Mesangial cell cultures were stimulated with MRC OX-44 antibody. DNA synthesis was measured by thymidine incorporation. Extracellular signal-regulated kinase (ERK) activation was determined by Western blot.

Results. CD53 was present in mesangial cells in vivo and in culture. Ligation of CD53 antigen with a monoclonal antibody triggered the induction of DNA synthesis, which was not sensitive to inhibitors of signaling pathways that use phosphatidylinositol 3-kinase (PI3K) and protein kinase C, or to calcium channel inhibitors, such as thapsigargin and verapamil. The DNA synthesis was inhibited by PD98059, a specific inhibitor of MEK that prevents ERK1/ERK2 activation. In addition, ERK1 and ERK2 activation by phosphorylation occurred following CD53 antigen ligation. The DNA synthesis was due to de novo synthesis and not to DNA repair as a consequence of the initiation of apoptosis, determined by flow cytometry, and lack of proteolytic activation of PARP by caspase 3. CD53 antigen ligation also induced an increase in mitochondrial activity.

Conclusions. To our knowledge this is the first identification of a tetraspanin protein in mesangial cells. CD53 antigen delivers a signal that initiates DNA synthesis. This signal is mediated by ERK1/ERK2 activation, but it is not sufficient to complete the cell cycle.

Key words: CD53, tetraspanin antigen, apoptosis, proliferation, mesangial cells, ERK, cell signaling.

Received for publication June 10, 2002
and in revised form August 7, 2002
Accepted for publication September 20, 2002

The extracellular matrix plays a major role in the organization of tissue structures. Different types of proteins, such as integrins, mediate the interactions between cells and the extracellular matrix (ECM), but new proteins that modulate these interactions have been identified, such as the tetraspanin proteins. Tetraspanin proteins constitute a group of integral membrane proteins defined by their structural characteristics, with four transmembrane domains and two extracellular loops, the second loop conferring their functional specificity [1, 2]. Currently there are 28 members that include several CD antigens (CD9, CD37, CD53, CD63, CD81, CD82, CD151), and they are present in different cell types such as lymphoid, epithelial and muscle cells. Some of them (CD53 and CD37) have a more restricted pattern of cell expression than others [1, 2], and in some tissues can form specific structures, such as uroplakin in the bladder [3]. Tetraspanin antigens have been detected either as free molecules or as interacting with other tetraspanin proteins [4, 5], organized in a macromolecular structure [2, 6]. Some of the tetraspanins also interact with integrins, particularly those containing the β1 subunit [2, 6, 7], major histocompatibility complex (MHC) class II antigens [8], T-cell receptor [9], CD19 molecules [10], members of the immunoglobulin superfamily [11], and some growth factor receptors, such as epidermal growth factor receptor (EGFR) and the hepatocyte EGF-like receptor [12].

The functions of tetraspanin proteins are not well characterized [13]. Tetraspanin proteins can influence several biological processes, such as cell motility [14] and homotypic adhesion [15–18]. In tumors, low levels of tetraspanin proteins are an indicator of high metastatic potential, and this effect is correlated with the modulation of cell motility [19]. Because of the complex pattern of tetraspanin gene expression, it is very likely that the adhesion and migration properties of tumors are conditioned by the alteration in the composition of cell-specific patterns.

The CD53 antigen expression is mainly restricted to the lymphoid-myeloid lineage, with very low levels in other cell types [1, 20]. CD53 is proteolytically down-regulated in human neutrophils stimulated with different chemotactic stimuli, such as platelet activating factor or N-formyl-methionyl-leucyl-phenylalanine (fMLP) peptide [21]. The CD53 ectopic expression might facilitate tumor migration by the lymphatic system or reflect a phenotype of resistance to radiation, as has been demonstrated by the overexpression of CD53 [22]. In addition, CD53 deficiency has a clinical phenotype similar to those of inherited defects of cell adhesion molecules, such as integrins [23].

Tetraspanin proteins are capable of sending intracellular signals by themselves. The CD53/OX44 antigen is the most characterized tetraspanin antigen in rat cells [24]. Ligation of the rat CD53 antigen induces nitric oxide production in rat macrophages [25] and homotypic adhesion in B-cell lymphomas [15]. The ligation of CD53 has been shown to induce intracellular calcium mobilization in different cell types, such as human B-cells and monocytes [26, 27] and rat macrophages [15, 25]. Ligation of the rat CD53 antigen mediates some signals through the phosphatidylinositol 3-inositol kinase (PI3K) and protein kinase C (PKC) [25, 28]. CD53 induces specific structures in homotypic cell adhesion [15], which might form part of the glycosynapse [29].

Renal mesangial cells are pericytes placed in between the glomerular capillary loops and are derived from the myeloid lineage. Many forms of renal diseases that progress to chronic renal failure are characterized by mesangial cell activation, proliferation and, more prominently, accumulation of mesangial extracellular matrix [30]. Factors that control mesangial cell function include cytokines and growth mediators, matrix components and interactions with other cells such as endothelial and epithelial cells. Understanding the regulation of mesangial proliferation is important for the design of therapeutic strategies to alleviate or arrest proliferative glomerular disease.

There are no data on the presence or role of tetraspanin proteins in mesangial cells. Because tetraspanin proteins can modulate cellular interactions with the extracellular matrix, as well as affect cell motility, the present study searched for the presence of the CD53 antigen in primary rat mesangial cells. In addition, we examined the effects of antigen ligation in order to determine if it has, by itself, any biological effect that would indicate its active participation in some aspects of mesangial cell biology, such as cell proliferation.

METHODS

Reagents and monoclonal antibodies

L-glutamine, penicillin, streptomycin sulfate, insulin, transferrin, selenium, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) and EGF were from Sigma (St. Louis, MO, USA). RPMI 1640 supplemented with 10% fetal calf serum (FCS) was from Flow Laboratories (Woodcock Hill, UK). The inhibitors thapsigargin, verapamil, genistein, bisindolylmaleimide, piceatannol, wortmannin and PD098059 were from Calbiochem (San Diego, CA, USA). [^{3}H]-thymidine was from Amersham Biosciences (Little Chalfont, UK). The biotin-streptavidin amplified detection system (ABC Immunostain Systems) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibody MRC OX44 (anti rat CD53) was from Serotec (Oxford, UK) and was used at 10 μg/mL based on previous stimulation studies [25]. The PC10 monoclonal antibody against proliferating cell nuclear antigen (PCNA; BD Biosciences, Heidelberg, Germany), the monoclonal antibody for PARP (Enzyme Systems Products, Livermore, CA, USA), and AC15 antibody for β-actin (Sigma) were used at 1:5000 dilutions for the Western blots. The mitogen activated protein (MAP) kinases ERK1 and ERK2 were detected with clone C-14 antibody from Santa Cruz Biotechnology, diluted 1:5000 for Western blot, and ERK1 and 2 phosphorylated in Tyr204 were detected with monoclonal antibody clone E4 (Santa Cruz) diluted 1:10,000 for Western blots.

Immunohistochemistry

Rats were anesthetized with ether and renal perfusion with ice-cold saline through the abdominal aorta was performed to wash the blood out from the remnant kidney. A piece of the kidney, including cortex and medulla, was trimmed and fixed by immersion in 4% buffered formalin for one day. The blocks thus obtained were dehydrated and embedded in paraffin, and 3 μm sections were cut and mounted on glass slides. An antigen retrieval process was carried out on slides with citrate solution (BioGenex, San Ramon, CA, USA) in oven for three minutes. Then the slides were washed in phosphate buffer and, after blocking endogenous peroxidase with hydrogen peroxide, incubated for 30 minutes at room temperature with the monoclonal antibody MRC OX44 at a 1:500 dilution, followed by a peroxidase label polymer amplified detection system (Envision+ System; Dako, Glostrup, Denmark). The chromogen used was 3,3′-diaminobencidine, and then the tissues were counterstained with hematoxylin. Omitting the specific antibody in the first incubation step controlled the specificity of the immunostaining.

Primary mesangial cell culture

Kidney glomeruli were isolated from Wistar rats by successive mechanical sieving (150 and 50 μm), treated with 300 U/mL collagenase, and plated in 35 mm plastic tissue culture dishes as previously described [31]. Cells were grown in RPMI 1640 supplemented with 10% FCS,

L-glutamine (1 mmol/L), penicillin (0.66 μg/mL), streptomycin sulfate (60 μg/mL), 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium and buffered with HEPES, pH 7.2. The culture medium was changed every two days. Studies were performed on day 21 or 22, at which time epithelial cells were no longer detected in the culture dishes. The identity of the cells was confirmed by morphological and functional criteria previously described [32].

DNA synthesis and cell proliferation

To determine cell proliferation, 15,000 mesangial cells were plated in each dish in 24-well plates [33]. After 48 hours, cells were starved by withdrawal of the serum, but maintaining the complements (insulin and sodium selenite). After 24 hours, 1 μCi of [^{3}H]-thymidine was added to the cells and incubated for an additional six hours. Afterwards the cells were washed with phosphate-buffered saline (PBS) and DNA was extracted by addition of cold trichloroacetic acid at 10% for 15 minutes. The supernatant was removed and the cells were incubated with 1 mL of NaOH at 60°C for 30 minutes with occasional shaking. From this solution 500 μL were mixed with scintillation liquid and the thymidine counts were measured in a Wallac1409 DSA scintillation counter (Perkin-Elmer, Warrington, UK). The radioactivity was normalized by determination of the protein concentration using a Protein assay kit (Bio-Rad, Richmond, CA, USA). Cellular proliferation also was determined by staining of cell nuclei with violet crystal as previously described [34]. Briefly, the cells were fixed with 10% glutaraldehyde for 10 minutes, and washed in PBS. Cell nuclei DNA was stained with a solution of 0.2% crystal violet for 30 minutes in darkness. The plates were left to air dry, 2 mL of acetic acid were added to the wells and the absorbance at 595 nm was measured.

Flow cytometry analysis

The cells were analyzed after staining with the appropriate monoclonal antibody or with propidium iodide in a FACScalibur flow cytometer (Becton-Dickinson, Lincoln Park, NJ, USA). The monoclonal antibody MRC OX44 against rat CD53 was used at a dilution of 1:100 for flow cytometry and at 1:50 for immunofluorescence. The secondary antibody was a FITC-labeled anti-IgG.

Protein extracts and Western blot analysis

Cells were washed in PBS and lysed in a buffer containing 150 mmol/L NaCl, 0.25 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 1 mmo/L NaF, 100 μmol/L Na orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and Tris-HCl 10 mmol/L, pH 8.0. For determination of the PCNA antigen the buffer also contained 0.1% sodium dodecyl sulfide (SDS) and 1% Na deoxycholate. Cell lysates were treated with 100 U/mL of DNAse I to eliminate DNA. For Western blots, between 20 and 100 μg of protein were loaded in the denaturing (7.5 to 10%) polyacrylamide gel. The proteins were transferred to a PVDF membrane, Immobilon-P from Millipore (Bedford, MA, USA). Membranes were blocked with 5% skimmed milk in PBS, and then incubated with the specific antibody, followed by the secondary antibody, a rabbit anti-mouse IgG with peroxidase. Both incubations were performed a room temperature for one hour. After washing the filters, the signal was developed with a chemiluminescence ECL kit from Amersham.

Mitochondrial activity assays

The mitochondrial activity associated to proliferation signals was determined using a cell proliferation kit from Roche Diagnostics (Méylan, France) based on the conversion of the XTT tetrazolium salt to formazan that correspond to cells metabolically active. Color development was determined in a spectrophotometer multiwell plate reader at 492 nm.

RESULTS

Expression of the OX44/CD53 antigen in mesangial cells

Mesangial cells are considered to originate from the myeloid lineage, with some smooth muscle and endothelial cell features, and therefore are likely to express some tetraspanin antigens related with this lineage, such as CD53. First we determined the presence of the CD53 antigen in kidney tissue sections. In rat kidney glomeruli, this antigen was detected in mesangial and with less intensity in endothelial cells (Fig. 1A). The presence and level of the CD53 antigen in rat primary cultures of mesangial cells was analyzed also by flow cytometry using the MRC OX44 monoclonal antibody, which was specific for the rat CD53 antigen [24]. Primary cultures of rat mesangial cells expressed the CD53 antigen as demonstrated by flow cytometry (Fig. 1B). The level of CD53 antigen appeared to be dependent on growth conditions. In cells deprived of FCS, and thus resting or with a low proliferation rate, there was a lower CD53 membrane levels than in cells proliferating in a medium with 10% FCS. Resting serum-starved cells had a more heterogeneous level of CD53 expression, as shown by its broader peak in flow cytometry analysis (Fig. 1C).

Stimulation of DNA synthesis by ligation of the CD53 antigen

Different types of membrane proteins can trigger a growth response in many cell types. Primary renal mesangial cells in situ have a slow growth rate under physiological conditions, probably due to the lack of adequate stimulation in its extracellular environment. Because ligation of tetraspanin antigens on the cell surface can

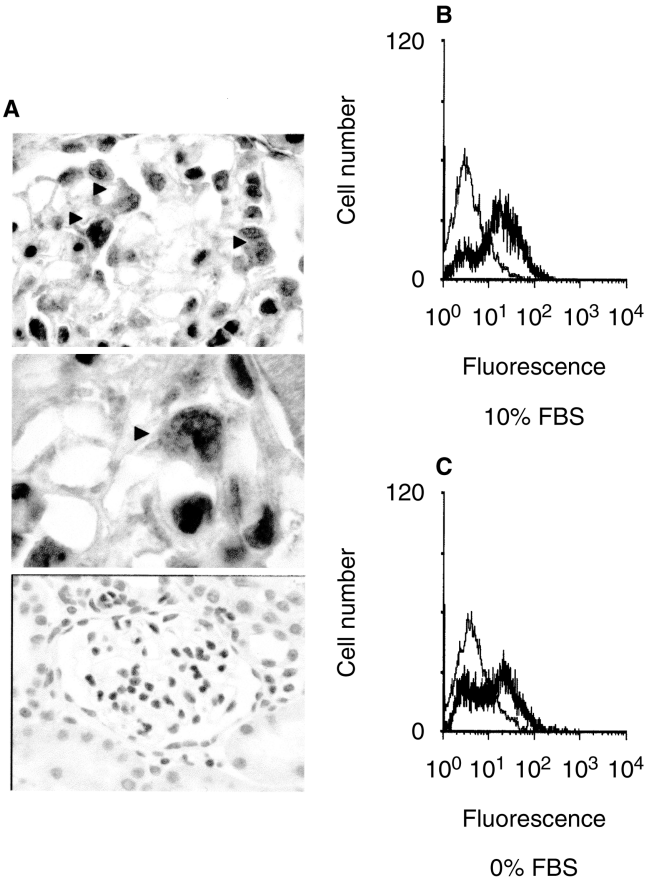

Fig. 1. CD53 expression in rat mesangial cells. Expression of CD53 by immunohistochemistry in rat kidney slides. In the glomeruli, CD53 expression is mostly expressed in mesangial cells (arrows) and more weakly in endothelial cells. At the top is a view of the field, in the middle a mesangial cell is shown and at the bottom is the negative control staining (*A*). Flow cytometry analysis of mesangial cells isolated from normal Wistar rat grown either in the presence of fetal calf serum (*B*) or deprived of serum (*C*). As the negative control, an isotype-matched antibody was used.

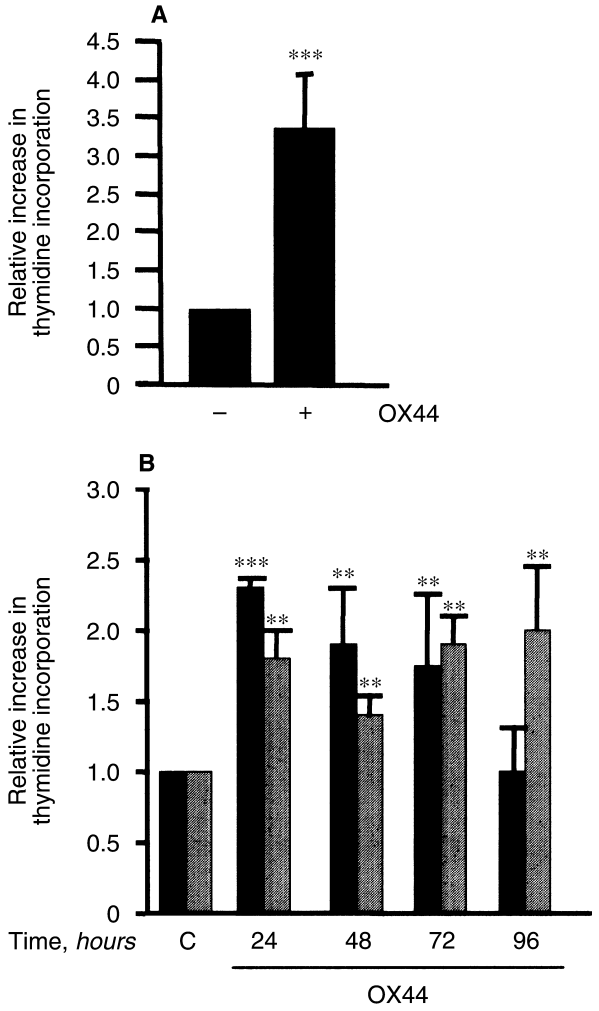

Fig. 2. Induction of DNA synthesis by CD53. (*A*) Induction of DNA synthesis in Wistar mesangial cells by ligation of the CD53 antigen with monoclonal antibody MRC OX44. (*B*) Effect of time on the incorporation of DNA synthesis induced by OX44 ligation in mesangial cells. The incorporation of thymidine was performed either as six hour pulses, the last six hours of each day (■), or as a continuous incorporation by the presence of thymidine from the start (▩), reflecting the overall metabolism, synthesis and degradation, of DNA. Results are the mean ± SD of four experiments (**$P < 0.01$; ***$P < 0.001$). Controls were in the presence of a non-specific antibody.

trigger cell growth in some cell types [35], we tested if the CD53 antigen in mesangial cells was able to generate a signal in response to its ligation with the MRC OX44 antibody, a situation that might mimic the engagement of this membrane protein by the extracellular matrix. As control, cells were similarly incubated with an isotype-matched non-reactive antibody. Before stimulation with the antibody the cell culture was synchronized by serum deprivation for 24 hours. The synchronization and accumulation of cells in the G1 phase was confirmed by flow cytometry (not shown). Next the antibody was added and its effect on [^{3}H]-thymidine incorporation to DNA was determined. CD53 antigen ligation induced an increase in [^{3}H]-thymidine incorporation, which might be a consequence of either DNA replication or DNA repair (Fig. 2A).

Next, DNA synthesis after CD53 ligation was determined in two different ways, either as daily six-hour [^{3}H]-thymidine pulses (between 18 to 24 hours of each day), or as a continuous [^{3}H]-thymidine incorporation. The pulse experiment showed that thymidine incorporation was much higher the first day (Fig. 2B), and there was a drop in the daily incorporation in the remaining days. These results suggested that after antibody addition there was a burst signal for DNA synthesis that was not maintained in the following days, where the incorporation appeared to drop. On the other hand, when [^{3}H]-thymidine was present all the time in the medium, thus reflecting the balance between DNA synthesis and degradation, there was an overall increase in its accumulation, but the peak in the first day also was detectable (Fig. 2B).

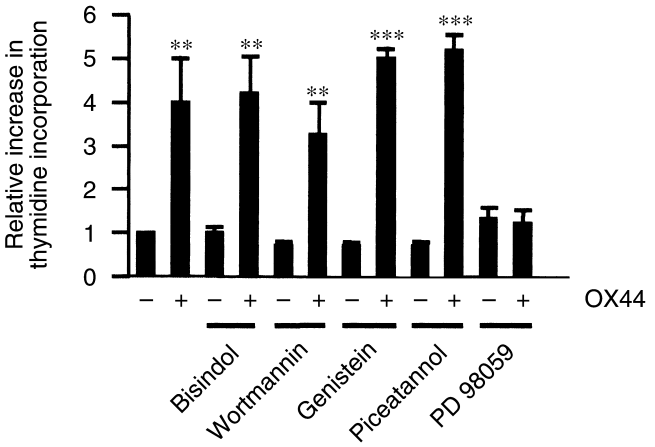

Fig. 3. Effects of signaling pathway inhibitors on de novo DNA synthesis induced by CD53 ligation in rat mesangial cells. The thymidine incorporation was determined in the presence of PKC inhibitor (bisindolylmaleimide at 100 nmol/L), PI3K inhibitor (wortmannin at 100 nmol/L), MEK inhibitor (PD 098059 at 10 μmol/L), and tyrosine kinases inhibitors (genistein at 10 ng/mL and piceatannol 100 nmol/L). The MEK kinase inhibitor prevented the incorporation of thymidine. Results are the mean ± SD of four experiments (**$P < 0.01$; ***$P < 0.001$).

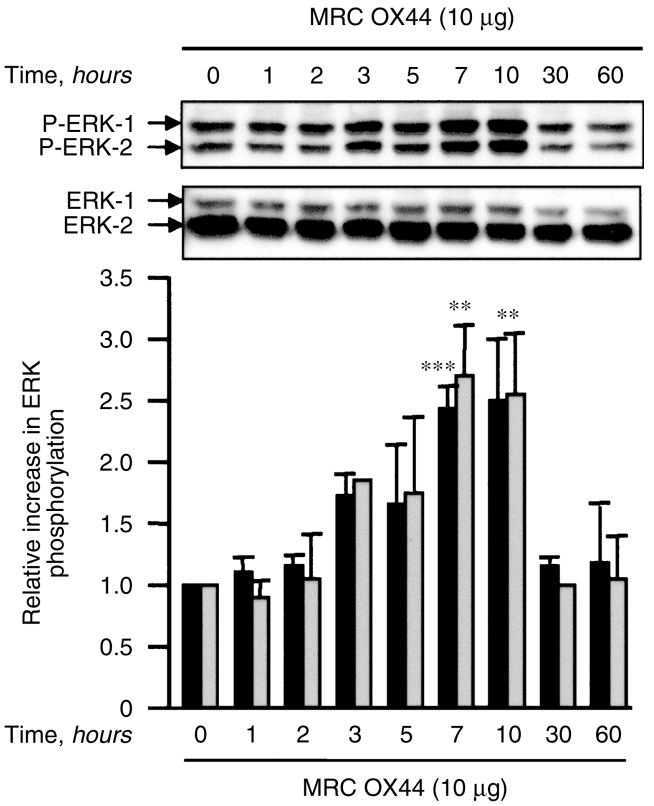

Fig. 4. Implication of ERK in the CD53 response. (*A*) Phosphorylation of ERK kinase induced by CD53 ligation. The proliferation signals were analyzed following addition of the antibody. The upper blot shows the detection of phosphorylated ERK1 and ERK2, and the lower blot shows the total amount of ERK1 and ERK2 in the cells detected with an anti-ERK antibody. (*B*) The normalization of phospho-ERK protein with respect to total ERK protein. Symbols are: (■) ERK1; (□) ERK2. Results are the mean ± SD of four experiments (**$P < 0.01$; ***$P < 0.001$).

Effects of inhibitors of signaling pathways on the DNA synthesis induced by CD53

In an attempt to identify the signaling pathways implicated in this proliferative response, several inhibitors with specificity for known intracellular signaling pathways were used. The inhibitors used were: wortmannin, an inhibitor of the phosphatidylinositol 3-kinase (PI3K) catalytic activity; genistein and piceatannol, two tyrosine kinase inhibitors; and bisindolylmaleimide, a competitive inhibitor of protein kinase C (PKC). All of them were used at the concentrations indicated in the Figure 3 legend, which minimized their potential toxicity with time, and have been used previously in other studies related with the rat CD53 antigen [15]. None of these inhibitors had any significant effect on the DNA synthesis induced by ligation of the CD53 antigen (Fig. 3). However, the signal triggered by CD53 ligation and its effect on DNA synthesis was completely abolished by the addition of PD 098059 (Fig. 3), a highly specific inhibitor of MAPK/ERK kinase (MEK), a kinase required for activation of extracellular signal-regulated kinase (ERK) by phosphorylation [36].

ERK is implicated in the response to CD53 antigen ligation

The use of specific kinase inhibitors suggested that the ERK family of MAPK was implicated in the signal triggered by CD53 ligation. There are two ERK proteins (1 and 2) that can be activated by phosphorylation in Tyr204. To confirm that the ERK1 and ERK2 indeed are activated by CD53 ligation, their specific phosphorylation in Tyr204 was determined. Extracts from stimulated and control cells were analyzed by Western blot with a specific antibody against ERK phosphorylated in residue Tyr204, and this signal was normalized with the level of ERK protein present in the extract. As shown in Figure 4, the ligation of CD53 with the monoclonal antibody (mAb) MRC OX44 induced a transient phosphorylation of both ERK1 and ERK2 that reached a maximum between 7 and 10 minutes after antibody addition. We conclude that CD53 ligation induces a stimulatory signal mediated by these kinases.

DNA synthesis is the result of an abortive progression of the cell cycle and not to DNA repair

To test if the increase in DNA synthesis after CD53 ligation was indeed the start of a proliferative response that did not progress any further, we analyzed the progression of the cell cycle under the same experimental conditions used in the flow cytometry studies. After the addition of the antibody there was a small increase in the S-G_2 phase that did not progress any further, and

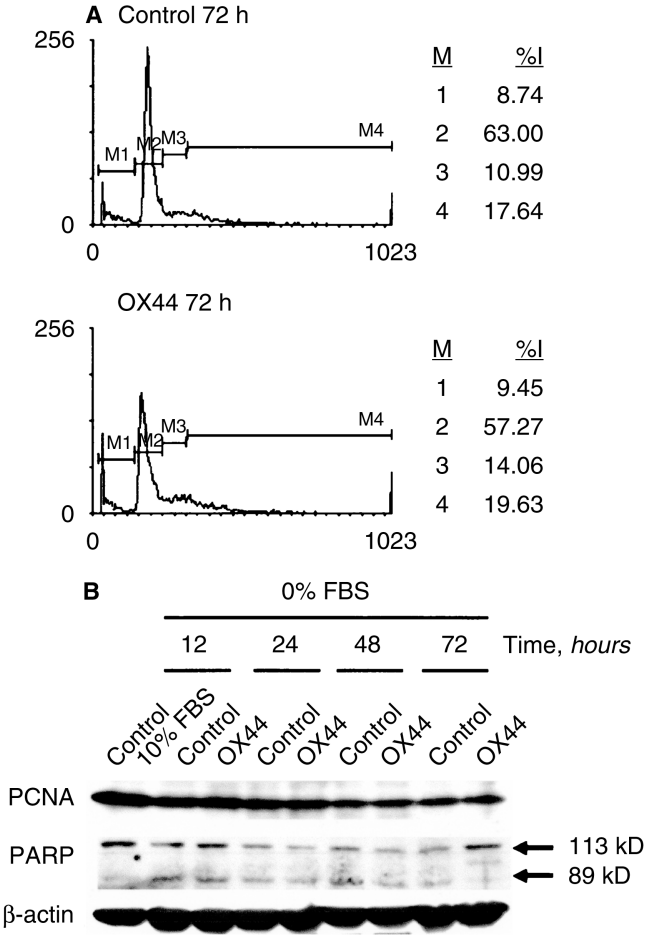

Fig. 5. Effects of CD53 ligation on cell cycle. (*A*) Flow cytometry analysis of the population of mesangial cells at 72 hours after antibody addition. The increased in the synthesis phase after antibody addition with respect to its control is indicated by the increase in the M_3 (S phase) + M_4 (G_2-M phase) windows, which combined represent a 5% increase, accompanied by a similar decrease in the M_2 window (G_1). (*B*) Western blots showing the level of PARP and PCNA antigens in control and OX44 treated cells. Gel loading was determined by performing a Western blot with an antibody specific for β-actin.

that in the end resulted in a minor increase in cell death, a typical response to a blockade of cell cycle progression. This was best illustrated by the flow cytometry pattern of propidium iodide staining at 72 hours after antibody addition. The decrease in the M_2 (G_1 phase) window (5% reduction, from 63 to 57) was accompanied by a similar increase in the M_3 + M_4 windows (S + G_2/M phases), from 28 to 34% (Fig. 5A) when the antibody was present. This observation indicates a minor increase in DNA synthesis, consistent with the findings in thymidine incorporation experiments.

Activation of the apoptotic pathway and subsequent DNA damage also could cause incorporation of thymidine in the partially degraded DNA, particularly at the beginning of the process as the cell attempts to repair it. To assess if apoptosis was triggered by MRC OX44 antibody, we determined the level of PARP, a protein of 113 kD that appears as a smaller band of 89 kD when caspase 3 has been activated. The level of 113 kD PARP protein and of its proteolytic fragment either did not change or was slightly smaller in cells treated with the antibody with respect to the control (Fig. 5B). The analysis of total DNA did not show the apoptotic ladder in cells treated with MRC OX44 antibody (not shown). Also, we determined the changes in PCNA (proliferating cell nuclear antigen), associated with DNA polymerase δ, and a component of nucleotide excision repair systems. The levels of PCNA were similar in mesangial cells stimulated with MRC OX44 antibody and in the controls (Fig. 5B), suggesting that there was not an increase in DNA repair in these cells. These observations suggest that the apoptotic pathway was not activated by ligation of the CD53 antigen.

Effect on cell proliferation

Mesangial cells have an irregular shape and have a heterogeneous distribution in culture that makes it difficult to count the cells. To confirm independently the effect induced by the ligation of CD53 and detected by flow cytometry, the total nuclear number in the mesangial cell culture dishes was determined using a colorimetric method based on staining by violet crystal and measuring the absorbance at 595 nm, which was proportional to the number of nuclei in the culture. The addition of MRC OX44 antibody induced an increase in absorbance with respect to control cells, which, while significant, did not represent an increase larger than 20% (Fig. 6A). This observation was consistent with the increase in DNA synthesis described earlier in this article.

Stimulation of mitochondrial activity

The level of mitochondrial activity is an indirect method for determining an increase in cell proliferation, since there is an increase in energy demand during proliferation. The mitochondrial activity of mesangial cells stimulated with MRC OX44 was determined using a colorimetric assay based on the determination of color development by degradation of tetrazolium salt. The addition of MRC OX44 to the culture medium produced an increase in the level of mitochondrial activity (Fig. 6B), which was also significant in a manner consistent with the data of flow cytometry and thymidine incorporation.

DISCUSSION

Mesangial cell proliferation is a major characteristic of several renal diseases that progress to end-stage renal failure [30]. Understanding the regulation of mesangial proliferation is important for the design of therapeutic strategies to alleviate or arrest proliferative glomerular disease. Among the factors that control mesangial cell

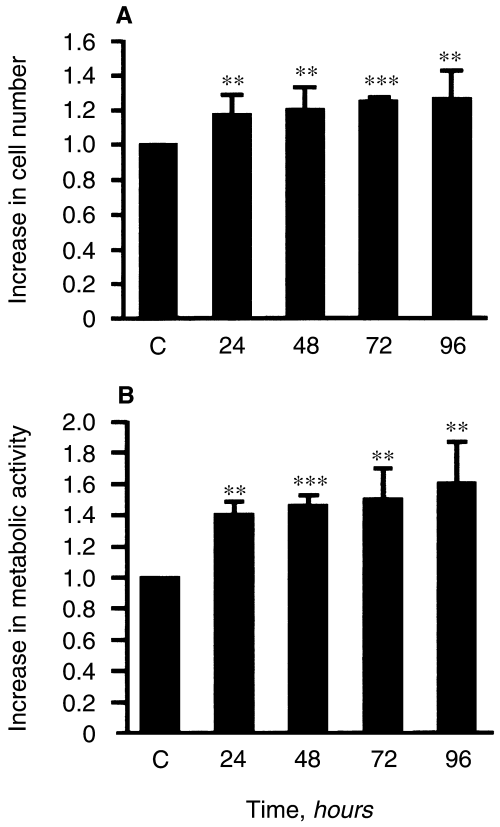

Fig. 6. Effects of OX44 ligation on cell number and mitochondrial activity of mesangial cells. (*A*) Increase in mesangial cell nuclei after OX44 ligation. Staining with crystal violet and measuring the absorbance at 595 nm quantitated the total amount of DNA. (*B*) Mitochondrial activity of Wistar mesangial cells determined with XTT. Results are the mean ± SD of four experiments (**$P < 0.01$; ***$P < 0.001$).

function are: cytokines, growth factors, mechanical stress, oxidative stress, high glucose, angiotensin II, endothelin, platelet activating factor, interaction with extracellular matrix components, and even drugs such as aminoglycosides [37]. Therefore, cultured mesangial cells constitute an important tool for studying pathophysiological events such as mesangial expansion, scarring, and glomerulosclerosis.

To date, there have been no published data on the possible role of tetraspanin proteins in mesangial cell biology. Our current report demonstrates the presence of one of these membrane proteins, CD53, in glomerular mesangial cells in situ and in primary cultures of rat mesangial cells. In addition, CD53 antigen ligation with its specific antibody MRC OX44 induces the initiation of DNA synthesis. This response is mediated by ERK1/ERK2 activation, as CD53 antigen ligation with its specific antibody induces the phosphorylation of ERK1 and ERK2, and the blockade of this pathway prevents from the MRC OX44-induced DNA synthesis. In addition, this response does not seem to be mediated by a tyrosine kinase receptor, as tyrosine kinase inhibitors have no effect on MRC OX44-induced DNA synthesis. These results open up a new type of pathway to ERK-mediated responses, but how the tetraspanin CD53 antigen transmits this signal to reach ERK1/2 is not known. The use of different types of inhibitors rules out pathways that are activated by CD53 ligation in other systems, such as PKC or PI3K [25, 38]. The ligation of CD53 antigen induces de novo gene expression, such as the inducible nitric oxide synthase in macrophages [25]. This effect is partly mediated by PKC, because CD53 ligation induces translocation of this kinase to the cell membrane and is sensitive to its inhibitors [25]. In addition, the physical association between tetraspanins and PKC was demonstrated [38], which necessarily has to be a secondary event following translocation of PKC to the plasma membrane, and thus is likely to be a consequence of the diacylglycerol induced by tetraspanin antigens [25, 39]. However, nothing is known yet about additional downstream components for the signals that originate in a tetraspanin antigen. In some lymphomas CD53 ligation also activates the N-terminal Jun kinase [40]. All these data combined suggest that CD53 can have a signaling role by itself in addition to its ability to modulate other signaling processes.

Because of the localization of tetraspanin proteins, and their interaction with other proteins, it was expected that they should have signaling capacity by themselves, and that they also should affect processes related to cell motility and proliferation. In renal mesangial cells we have shown that the ligation of the CD53 antigen is capable of initiating the de novo synthesis of DNA, but this signal does not result in progression of the cell cycle. In other cell types, such as a rat B-cell lymphoma [15, 28] and macrophages [25], as well as in human B and T-cell lymphomas [40], the ligation of the CD53 antigen is capable of signaling by itself, independently of their protein-protein interactions. Thus, ligation of rat CD53 can induce homotypic cell adhesion, a process that requires de novo protein synthesis [15]. Ligation of human CD53 has been shown to induce initiation of the G_1 phase of the cell cycle in B-cells [26]. In that latter situation, additional signals are required to complete the progression through the cell cycle.

Many proliferation signals are mediated by the activation of the ERK pathway, most of them in responses mediated by receptors with tyrosine kinase activity [41]. Proliferative responses in renal mesangial cells are triggered by a variety of receptor proteins, and some of them have been demonstrated to be mediated by the ERK pathway. Among them are the chemokine receptor CXCR3 [42], EGF [43], PDGF [44], bradykinin [45] or endothelin [46]. ERK-mediated proliferative responses of mesangial cells also occurred in situations of mechanical stress, such as stretching [47], in the presence of glycated albumin [48] or in hyperglycemia [47]. Transient activation of ERK

is related with proliferative signals, while sustained ERK stimulation result in cell differentiation [49]. It has been proposed that cell signaling mediated by ERK and initiated in the cell membrane are the result of signaling complexes that are anchorage dependent and have been called the signalosome [50]. In this context, tetraspanin proteins, because of their ability to organize signaling complexes on the cell membrane, which includes growth factor receptors and integrins, might represent the outward face of the signalosome that interacts with the extracellular matrix. The signal initiated in CD53 is likely to be a survival signal indicating proper interaction with the extracellular matrix rather than a growth signal. With respect to the possible role of CD53 in glomerular pathology, our preliminary results demonstrate an increase in CD53 expression in proliferating mesangial cells from hypertensive rats, suggesting that CD53 could be involved in glomerular pathologies associated to mesangial cell proliferation.

In summary, the present study demonstrates the presence of a member of the tetraspanins family, CD53, in rat mesangial cells in kidney sections and in primary cultures of rat mesangial cells. In addition, ligation of the CD53 antigen induces the initiation of DNA synthesis and this response is mediated by ERK1/ERK2 activation. The induction of a moderate proliferation induced by CD53 ligation in association with its possible effects on adhesion properties can contribute to a change in the organization of mesangial cells in the kidney, whose derangement might contribute the glomerular pathology where mesangial cells are implicated.

ACKNOWLEDGMENTS

M.Y. was supported by a fellowship from Instituto de Salud Carlos III. This work was funded by grants from Ministerio de Ciencia y Tecnología (SAF2000-0163 to P.A.L. and SAF2001-1701 to J.M.L-N) and Junta de Castilla y León (CSI1/01 to P.A.L., and SA89/02 to J.M.L-N.).

Reprint requests to Dr. P.A. Lazo, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain.
E-mail: plazozbi@usal.es

APPENDIX

Abbreviations used in this article are: ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular-regulated kinase; fMLP, N-formyl-methionyl-leucyl-phenylalanine; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PARP, poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; TGF-β, tumor growth factor-β.

REFERENCES

1. Maecker HT, Todd SC, Levy S: The tetraspanin superfamily: Molecular facilitators. (review) *FASEB J* 11:428–442, 1997
2. Boucheix C, Rubinstein E: Tetraspanins. *Cell Mol Life Sci* 58: 1189–1205, 2001
3. Walz T, Haner M, Wu X-R, *et al*: Towards the molecular architecture of the asymmetric unit membrane of the mammalian urinary bladder epithelium: A closed "twisted ribbon" structure. *J Mol Biol* 248:887–900, 1995
4. Mannion BA, Berditchevski F, Kraeft S, *et al*: Transmembrane-4-superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associate with integrin a4b1 (CD49d/CD29). *J Immunol* 157:2039–2047, 1996
5. Rubinstein E, La Naour F, Lagaudriere-Gesbert C, *et al*: CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. *Eur J Immunol* 26:2657–2665, 1996
6. Berditchevski F: Complexes of tetraspanins with integrins: More than meets the eye. *J Cell Sci* 114:4143–4151, 2001
7. Hemler ME: Specific tetraspanin functions. *J Cell Biol* 155:1103–1108, 2001
8. Angelisova P, Hilgert I, Horejsi V: Association of four antigens of the tetraspan family (CD37, CD53, TAPA-1, and R2/C33) with MHC class II glycoproteins. *Immunogenetics* 39:249–256, 1996
9. Damjanovich S, Matko J, Matyus L, *et al*: Supramolecular receptor structures in the plasma membrane of lymphocytes revealed by flow cytometric energy transfer, scanning force- and transmission electron-microscopic analyses. *Cytometry* 33:225–233, 1998
10. Horvath G, Serru V, Clay D, *et al*: CD19 is linked to the integrin-associated tetraspans CD9, CD81, and CD82. *J Biol Chem* 273: 30537–30543, 1998
11. Stipp CS, Kolesnikova TV, Hemler ME: EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. *J Biol Chem* 276:40545–40554, 2001
12. Shi W, Fan H, Shum L, Derynck R: The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. *J Cell Biol* 148:591–602, 2000
13. Boucheix C, Duc GHT, Jasmin C, Rubinstein E: Tetraspanins and malignancy. *Exp Rev Mol Med* 31 January, http://www-ermm.cbcu.cam.ac.uk/01002381h.htm, 2001
14. Higashiyama M, Taki T, Ieki Y, *et al*: Reduced motility related protein-1 (MRP1/CD9) gene expression as a factor of poor prognosis in non-small cell lung cancer. *Cancer Res* 55:6040–6044, 1995
15. Lazo PA, Cuevas L, Gutierrez del Arroyo A, Orue E: Ligation of CD53/OX44, a tetraspan antigen, induces homotypic adhesion mediated by specific cell-cell interactions. *Cell Immunol* 178:132–140, 1997
16. Levy S, Todd SC, Maecker HT: CD81 (TAPA-1): A molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol* 16:89–109, 1998
17. Shibagaki N, Hanada K, Yamashita H, *et al*: Overexpression of CD82 on human T cells enhances LFA-1 / ICAM-1–mediated cell-cell adhesion: Functional association between CD82 and LFA-1 in T cell activation. *Eur J Immunol* 29:4081–4091, 1999
18. Lagaudriere-Gesbert C, La Naour F, Lebel-Binay S, *et al*: Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: Only CD9 upregulates HB-EGF activity. *Cell Immunol* 182: 105–112, 1997
19. Ikeyama S, Koyama M, Yamaoko M, *et al*: Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP1/CD9) DNA. *J Exp Med* 177:1231–1237, 1993
20. Horejsi V, Vlcek C: Novel structurally distinct family of leucocyte surface glycoproteins including CD9, CD37, CD53 and CD63. *FEBS Lett* 288:1–4, 1991
21. Mollinedo F, Martin-Martin B, Gajate C, Lazo PA: Physiological activation of human neutrophils downregulates CD53 cell surface antigen. *J Leuk Biol* 63:699–703, 1998
22. Voehringer DW, Hirschberg DL, Xiao J, *et al*: Gene microarray identification of redox and mitochondrial elements that control resistance or sensitivity to apoptosis. *Proc Natl Acad Sci USA* 97:2680–2685, 2000
23. Mollinedo F, Fontán G, Barasoaín I, Lazo PA: Recurrent infectious diseases in human CD53 deficiency. *Clin Diag Lab Immunol* 4:229–231, 1997
24. Bellacosa A, Lazo PA, Bear SE, Tsichlis PN: The rat leukocyte antigen MRC OX-44 is a member of a new family of cell surface

glycoproteins which appears to be involved in growth regulation. *Mol Cell Biol* 11:2864–2872, 1992
25. Boscá L, Lazo PA: Induction of nitric oxide release by MRC OX-44 (anti-CD53) through a protein kinase C-dependent pathway in rat macrophages. *J Exp Med* 179:1119–1126, 1994
26. Olweus J, Lund-Johansen F, Horejsi V: CD53, a protein with four membrane-spanning domains, mediates signal transduction in human monocytes and B cells. *J Immunol* 151:707–716, 1993
27. Rasmussen A, Blomhoff HK, Stokke T, *et al*: Cross-linking of CD53 promotes activation of resting human B lymphocytes. *J Immunol* 153:4997–5007, 1994
28. Barcia R, García-Vargas S, Bosca L, Lazo PA: CD53 antigen and epidermal growth factor induce similar changes in the pattern of phorbol ester binding in a B cell lymphoma. *Cell Immunol* 169: 107–112, 1996
29. Hakomori SI: Inaugural article: The glycosynapse. *Proc Natl Acad Sci USA* 99:225–232, 2002
30. Johnson RJ: The glomerular response to injury: Progression or resolution? *Kidney Int* 45:1769–1782, 1994
31. Rodriguez-Puyol D, Lamas S, Olivera A, *et al*: Actions of cyclosporine A on cultured rat mesangial cells. *Kidney Int* 35:632–637, 1989
32. Olivera A, Lopez-Novoa JM: Effect of adenosine and adenosine analogues on cyclic AMP accumulation in cultured mesangial cells and isolated glomeruli of the rat. *Br J Pharmacol* 107:341–346, 1992
33. MacLaughlin M, Martinez-Salgado C, Eleno N, *et al*: Adenosine activates mesangial cell proliferation. *Cell Signal* 9:59–63, 1997
34. Montero A, Rodriguez-Barbero A, Lopez-Novoa JM: A role for platelet-activating factor in endothelin-1-induced rat mesangial cell proliferation. *Eur J Pharmacol* 243:235–240, 1993
35. Van Compernolle SE, Levy S, Todd SC: Anti-CD81 activates LFA-1 on T cells and promotes T cell-B cell collaboration. *Eur J Immunol* 31:823–831, 2001
36. Davies SP, Reddy H, Caivano M, Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105, 2000
37. Mene P: Mesangial cell cultures. *J Nephrol* 14:198–203, 2001
38. Zhang XA, Bontrager AL, Hemler ME: Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J Biol Chem* 276:25005–25013, 2001
39. Berditchevski F, Tolias KF, Wong K, *et al*: A novel link between integrins, transmembrane-4-superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J Biol Chem* 272:2595–2598, 1997
40. Yunta M, Oliva JL, Barcia R, *et al*: Transient activation of the c-Jun N-terminal kinase (JNK) activity by ligation of the tetraspan CD53 antigen in different cell types. *Eur J Biochem* 269:1012–1021, 2002
41. Brunet A, Pages G, Pouyssegur J: Constitutively active mutants of MAP kinase kinase (MEK1) induce growth factor-relaxation and oncogenicity when expressed in fibroblasts. *Oncogene* 9:3379–3387, 1994
42. Bonacchi A, Romagnani P, Romanelli RG, *et al*: Signal transduction by the chemokine receptor CXCR3: Activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. *J Biol Chem* 276: 9945–9954, 2001
43. Grewal JS, Luttrell LM, Raymond JR: G protein-coupled receptors desensitize and down-regulate epidermal growth factor receptors in renal mesangial cells. *J Biol Chem* 276:27335–27344, 2001
44. Arar M, Xu YC, Elshihabi I, *et al*: Platelet-derived growth factor receptor beta regulates migration and DNA synthesis in metanephric mesenchymal cells. *J Biol Chem* 275:9527–9533, 2000
45. El-Dahr SS, Dipp S, Baricos WH: Bradykinin stimulates the ERK→Elk-1→Fos/AP-1 pathway in mesangial cells. *Am J Physiol* 275: F343–F352, 1998
46. Foschi M, Franchi F, Han J, *et al*: Endothelin-1 induces serine phosphorylation of the adaptor protein p66Shc and its association with 14–3-3 protein in glomerular mesangial cells. *J Biol Chem* 276: 26640–26647, 2001
47. Dlugosz JA, Munk S, Kapor-Drezgic J, *et al*: Stretch-induced mesangial cell ERK1/ERK2 activation is enhanced in high glucose by decreased dephosphorylation. *Am J Physiol Renal Physiol* 279: F688–F697, 2000
48. Cohen MP, Shea E, Shearman CW: ERK mediates effects of glycated albumin in mesangial cells. *Biochem Biophys Res Commun* 283:641–643, 2001
49. Schaeffer HJ, Weber MJ: Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. *Mol Cell Biol* 19:2435–2444, 1999
50. Howe AK, Aplin AE, Juliano RL: Anchorage-dependent ERK signaling - mechanisms and consequences. *Curr Opin Genet Dev* 12:30–35, 2002